Leading Edge
Review
Cell

Modifications of Small RNAs and Their Associated Proteins

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DOI 10.1016/j.cell.2010.11.018

Small regulatory RNAs and their associated proteins are subject to diverse modifications that can impinge on their abundance and function. Some of the modifications are under the influence of cellular signaling, thus contributing to the dynamic regulation of RNA silencing.

Introduction

The past decade has witnessed an explosion of research on small regulatory RNAs that has yielded a basic understanding of the many types of small RNAs in diverse eukaryotic species, the protein factors involved, and the functions of key factors along the RNA silencing pathways. Much more remains to be learned, however, with recent studies unveiling interesting new layers of regulation and complexity associated with small RNAs. We now know that both small RNAs and their associated protein factors can be modified at multiple steps in their biogenesis and effector pathways.

Insight into modifications of small RNAs came initially from sequencing efforts, which made it clear that most microRNA (miRNA) loci generate multiple isoforms (called isomiRs) apart from the reference sequence (Morin et al., 2008). Alternative/inaccurate processing partly explains the heterogeneity, but a substantial portion of the variation is due to RNA modifications. Small RNAs are modified either internally or externally by untemplated nucleotide addition, exonucleolytic trimming, 2′-O-methyl transfer, and RNA editing. Protein factors in RNA silencing pathways are also subject to various posttranslational modifications, including phosphorylation, hydroxylation, ubiquitination, and methylation. In this Review, we focus on the recent developments in the modifications of RNAs and proteins in RNA silencing pathways.

Small RNA Biogenesis

RNA silencing is a widespread mechanism of gene regulation in eukaryotes. At the core of all RNA silencing pathways lie small RNAs (20–30 nt in length) associated with the Argonaute family proteins (Kim et al., 2009). Small RNAs provide the specificity of regulation by base-pairing to the target nucleic acids while the Argonaute proteins execute the silencing effects. The Argonaute (Ago) proteins are grouped into Ago and Piwi subfamilies, and in animals, three types of small RNAs have been described: microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs).

miRNAs (~22 nt) induce mRNA degradation and/or translational repression. Nucleotides 2–7, from the 5′ end of the miRNA, are referred to as the "seed" and are critical for hybridization to the targets (Bartel, 2009). As a class, miRNAs are found in all tissues, although each miRNA species displays a unique spatio-temporal pattern of expression. An miRNA originates from a long primary transcript (pri-miRNA) containing a local hairpin structure (Kim et al., 2009). In animals, the nuclear RNase III Drosha liberates the hairpin-shaped precursor miRNA (pre-miRNA) (Figure 1). The cytoplasmic RNase III Dicer removes the terminal loop to produce a small RNA duplex, consisting of the functional miRNA strand and the passenger (*) strand (miRNA/miRNA*). The duplex then binds to the Argonaute loading complex (comprised of Dicer, TRBP, and Ago), whose action leads to the incorporation of the functional miRNA strand (mature miRNA) into Ago. The plant miRNA system differs from its animal counterparts in several aspects (Figure 2). The plant homolog of Dicer, Dicer-like 1 (DCL1), cleaves both pri-miRNA and pre-miRNA in the nucleus. Plant miRNAs generally show extensive complementary to their target mRNAs and induce endonucleolytic cleavage of the targets.

Endogenous siRNAs (endo-siRNAs, ~21 nt) are similar to miRNAs in their binding to the Ago subfamily proteins, in their dependence on Dicer for biogenesis, and in exerting their regulatory effects posttranscriptionally (Kim et al., 2009). But unlike miRNAs, endo-siRNAs originate from long double-stranded RNA precursors (dsRNAs), and their biogenesis does not require processing by Drosha. Endo-siRNAs are abundant in lower eukaryotes and in plants, whereas in mammals, they are found in restricted tissues such as the ovary.

Piwi-interacting RNAs (piRNAs, 21–30 nt) associate with the Piwi subfamily of Argonaute proteins. piRNAs mediate the silencing of repetitive elements in gonads via transcriptional and posttranscriptional silencing mechanisms. Production of piRNAs is not dependent on RNase III nucleases, and the steps and factors involved in their biogenesis remain largely unknown.

Modifications of Small RNAs

3′ End Modifications: Uridylation, Adenylation, and 2′-O-Methylation

The 3′ ends of mature miRNAs are highly heterogeneous, whereas the 5′ ends are relatively invariable. The patterns and sources of heterogeneity seem to vary depending on the miRNA species and the cell types. The 3′ end often contains extra 1–3 nucleotides that do not match the genomic DNA sequences. These untemplated nucleotides are added by

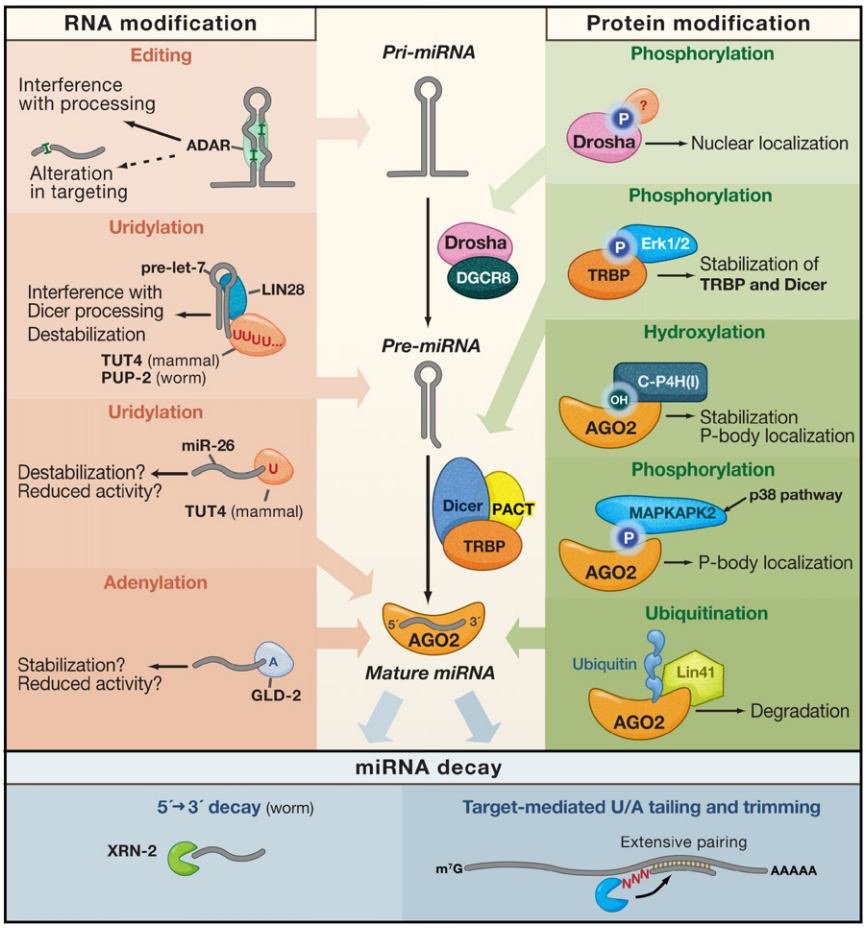

Figure 1. Modifications in the Animal MicroRNA Pathway

(Left) MicroRNAs (miRNAs) are subject to diverse modifications. Pri-miRNAs are edited by ADARs, which convert adenosine to inosine (I). RNA editing inhibits processing and/or alters target specificity. Pre-let-7 is regulated through uridylation. Lin28 recognizes pre-let-7 and, in turn, recruits a nucleotidyl transferase TUT4 (mammal) or PUP-2 (worms), which adds an oligo-uridine tail at the 3′ end of RNA. The uridylated pre-miRNA is resistant to Dicer processing and subject to decay. TUT4 also uridylates mature miRNA (miR-26), which reduces miRNA activity. Another nucleotidyl transferase GLD-2 adenylates mature miRNAs, which reduces the activity of miRNA and/or increases the stability of specific miRNAs (such as miR-122). (Bottom) Mature miRNAs are degraded through several mechanisms. In worms, a 5′→3′ exonuclease XRN-2 degrades miRNAs that are released from Ago. In flies and humans, extensive pairing between miRNA/siRNA and target RNA triggers tailing as well as 3′→5′ trimming of miRNA/siRNA. (Right) Protein factors, which are involved in the miRNA pathway, are also subject to various post-translational modifications. Human Drosha is phosphorylated at two serine residues, S300/S302, by an unknown kinase. Phosphorylation localizes Drosha to the nucleus, where the pri-miRNA processing occurs. MAP kinases Erk1/2 phosphorylate human TRBP at S142, S152, S283, and S286, which increases the protein stability of TRBP and Dicer. Ago2 is regulated by multiple modifications. A prolyl hydroxylase C-P4H(I) hydroxylates P700 in human Ago2, which enhances stability of Ago2 and increases P body localization. Phosphorylation of human Ago2 at S387 by MAPKAPK2, which is induced by p38 pathway, also promotes P body localization of Ago2. However, the biological significance of P body localization of Ago2 remains unclear. In mice, a stem cell-specific E3 ligase, mLin41, ubiquitinates Ago2 and targets it for proteosome-dependent degradation.

terminal nucleotidyl transferases that preferentially introduce uridyl or adenyl residues to the 3′ terminus of RNA.

The first indication of 3′ end modification of small RNA came from a *hen1* mutant of *Arabidopsis* (Li et al., 2005). HEN1 is a methyl transferase that adds a methyl group to the 2′-OH at the 3′ end of RNA (Yu et al., 2005). In *hen1* mutants, miRNAs are reduced in abundance and become heterogeneous in size due to uridylation at the 3′ end. Because U tailing correlates with the exonucleolytic degradation of mRNAs (Shen and Goodman, 2004), it was postulated that uridylation induces degradation of plant miRNAs and that the 2′-O-methyl moiety is required to protect small RNAs from uridylation and decay (see below). Consistent with this notion, in green algae *Chlamydomonas*, a nucleotidyl transferase, MUT68, uridylates the 3′ end of small RNA, and the RRP6 exosome subunit facilitates small RNA decay in a manner dependent on MUT68 in vitro (Ibrahim et al., 2010). Deletion of MUT68 results in elevated miRNA and siRNA levels, indicating that MUT68 and RRP6 collaborate in the turnover of mature small RNAs in plants.

Similar links between 2′-O-methylation, uridylation, and decay appear to exist in animals. A recent study on the zebrafish Hen1 homolog shows that piRNAs are uridylated and adenylated and that piRNA levels are reduced in *hen1* mutant germ cells (Kamminga et al., 2010). In flies and mice, piRNAs are methylated by HEN1 orthologs, but the connection to stability control remains unclear (Horwich et al., 2007; Kirino and Mourelatos, 2007; Ohara et al., 2007; Saito et al., 2007). In flies, dAgo2-bound RNAs (mostly siRNAs) are protected by 2′-O-methylation from being uridylated/adenylated, which in turn induces 3′ exonucleolytic trimming (Ameres et al., 2010). In nematode worms, the role of 2′-O-methylation has yet to be determined. However, a subset of endo-siRNAs associated with an Ago homolog CSR-1 is uridylated at the 3′ end, and the uridyl transferase CDE-1 (also known as CID-1 or PUP-1) negatively regulates these siRNAs, indicating that uridylation serves as a trigger for decay (van Wolfswinkel et al., 2009).

Although mature miRNAs lack methylation in animals, uridylation plays a significant role in the control of miRNA biogenesis. In mammalian embryonic stem cells, let-7 biogenesis is suppressed by the Lin28 protein that binds to the terminal loop of the let-7 precursors (Heo et al., 2008; Newman et al., 2008; Rybak et al., 2008; Viswanathan et al., 2008). Of interest, Lin28 induces 3′ uridylation of pre-let-7 by recruiting the terminal nucleotidyl transferase TUT4 (also known as ZCCHC11) (Hagan

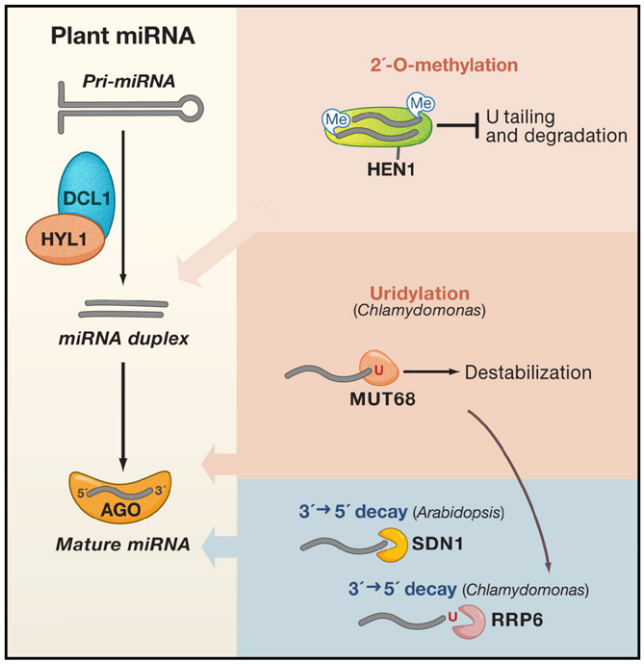

Figure 2. RNA Modifications in the Plant miRNA Pathway
In plants, both pri-miRNA and pre-miRNA are cleaved by DCL1/HYL1 complex. After cleavage, 3′ ends of miRNA duplex are 2′-O-methylated by a methyl transferase HEN1. The methylation protects miRNAs from uridylation and exonucleolytic degradation. In the green algae *Chlamydomonas*, the nucleotidyl transferase MUT68 attaches uridine residues at the 3′ end of mature miRNA lacking a methyl group. Then, the RRP6 exosome subunit, a 3′-to-5′ exonuclease, degrades the uridylated miRNAs. In *Arabidopsis*, a 3′→5′ exonuclease SDN1 is reported to degrade mature miRNAs.

et al., 2009; Heo et al., 2009). The oligo U-tail added by TUT4 blocks Dicer processing and facilitates the decay of pre-let-7. The homologs of TUT4 may have related functions in other organisms. In nematode worms, PUP-2 uridylates pre-let-7 in vitro and suppresses the let-7 function in vivo (Lehrbach et al., 2009).

Let-7 is unlikely to be the only miRNA uridylated at the pre-miRNA level. In support of this notion, untemplated 3′ uridine is frequently found in other mature miRNAs originating from the 3′ arm of pre-miRNAs (but significantly less frequently in those from the 5′ arm) (Burroughs et al., 2010; Chiang et al., 2010). Because untemplated uridylation is observed in cells lacking Lin28, it will be interesting to determine which pre-miRNAs other than pre-let-7 are controlled by uridylation and to identify additional factors required for pre-miRNA uridylation.

Although uridylation is generally thought to induce the decay of small RNAs, adenylation may have the opposite consequence. In cottonwood *P. trichoacarpa*, many miRNA families are adenylated at their 3′ ends, and adenylation prevents miRNA degradation in in vitro decay assay (Lu et al., 2009). In the case of mammalian miR-122, which is adenylated by cytoplasmic poly (A) polymerase GLD-2 (or TUTase2), 3′ end adenylation is also implicated in its stabilization (Katoh et al., 2009). In the liver of *Gld-2* knockout mice, the steady-state level of mature miR-122 is reduced, and the abundance of target mRNAs of miR-122 increases.

However, a recent study indicates that GLD-2 adenylates most miRNAs, and the adenylation may affect their activity rather than stability (Burroughs et al., 2010). Deep sequencing of Ago-associated small RNAs shows that adenylated miRNAs are relatively depleted in the Ago2 and Ago3 complexes, suggesting that adenylation may interfere with Ago loading. Similarly, it has been reported that uridylation of mature miR-26 by TUT4 results in the reduction of miR-26's activity without altering the miRNA levels (Jones et al., 2009). Therefore, it remains an interesting but yet unresolved issue whether or not uridylation/adenylation affects the stability of miRNAs in animals. One may speculate that 3′ modified miRNAs enter the silencing complex with altered frequencies, which in turn affects the small RNA's sensitivity to nucleases. Further examination is needed to identify the players involved in these processes, particularly the nucleases that recognize a U/A tail, and to dissect their action mechanisms.

miRNA Decay

Several nucleases degrade small RNAs (Figures 1 and 2). An *Arabidopsis* enzyme SDN1 (small RNA degrading nuclease, a 3′-to-5′ exonuclease) degrades single-stranded miRNAs in vitro (Ramachandran and Chen, 2008). miRNAs accumulate in a mutant lacking SDN1 and its related nucleases SDN2 and SDN3, indicating that the SDN proteins may act redundantly to degrade plant miRNAs. The 2′-O-methyl group at the 3′ end of miRNAs, which is a general feature of plant miRNAs, has a protective effect against SDN1 in in vitro assays. Of note, uridylation causes a small but detectable protective effect in the same in vitro assay, indicating that SDN1 is unlikely to be the nuclease responsible for U-tail-promoted degradation. Given that RRP6 (a 3′-to-5′ exonuclease) facilitates decay of small RNAs in a MUT68-dependent manner in *Chlamydomonas* extracts, multiple enzymes may be involved in small RNA decay in plants, playing partially overlapping but differential roles (Ibrahim et al., 2010).

In *C. elegans*, XRN-2 (a 5′-to-3′ exonuclease) is involved in the degradation of mature miRNAs (Chatterjee and Grosshans, 2009). Because miRNAs are tightly bound to and protected by Ago, it is unclear how XRN-2 accesses the 5′ end of an miRNA for decay. Of interest, larval lysate promotes efficient release of miRNA in vitro, implicating an as yet unknown factor that assists the release of miRNA from the otherwise tightly associated Argonaute protein (Chatterjee and Grosshans, 2009). In *Arabidopsis*, two XRN-2 homologs, XRN2 and XRN3, degrade the loop of miRNA precursor following processing, but they do not affect mature miRNA levels (Gy et al., 2007).

In mammals, a general nuclease for miRNAs has yet to be identified. Knockdown of XRN-1 or an exosome subunit in human cells results in only partial upregulation of miR-382, and XRN-2 depletion does not have a significant effect (Bail et al., 2010). Thus, it awaits further investigation whether or not there is one major conserved pathway for miRNA decay in mammals.

There have been intriguing reports of regulated decay of miRNAs. For instance, miR-29b is degraded in dividing cells more rapidly than in mitotically arrested cells (Hwang et al., 2007). In the central nervous system of *Aplysia*, the levels of miR-124 and miR-184 decrease in 1 hr after treatment with the neurotransmitter serotonin (Rajasethupathy et al., 2009).

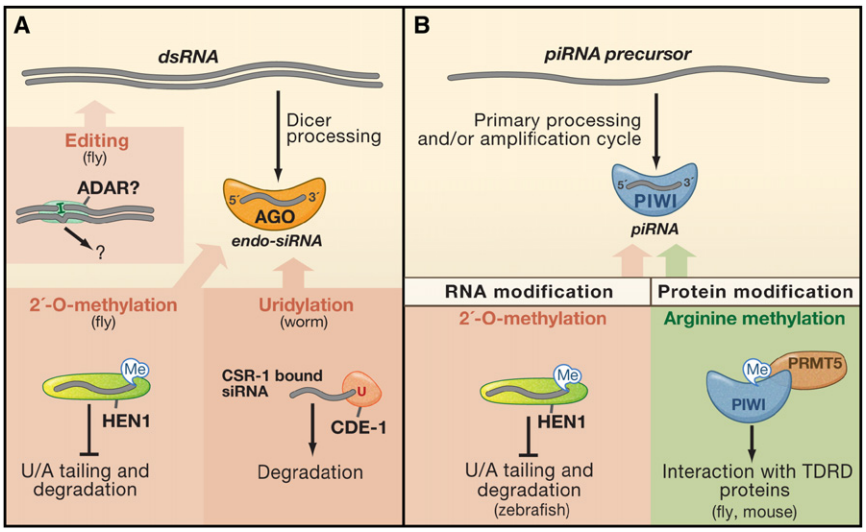

Figure 3. Modifications in the Endo-siRNA and piRNA Pathways

(A) Endogenous small interfering RNAs (endo-siRNAs) are processed from long dsRNAs in a Dicer-dependent manner and are loaded onto Ago proteins. High-throughput sequencing data show that the adenosine-to-inosine (I) editing occurs in fly endo-siRNAs, likely by ADAR, although the role of RNA editing is unknown. Fly endo-siRNAs bound to dAgo2 are 2′-O-methylated by HEN1 homolog, which protects RNAs from uridyl/adenyl tailing and degradation. In worms, a subset of endo-siRNAs, which are associated with an Ago homolog CSR-1, is uridylated at the 3′ end by the nucleotidyl transferase CDE-1. (B) piRNAs are generated from single-stranded RNA precursors that are processed by primary processing and/or secondary processing (ping-pong amplification cycle). piRNAs are associated with Piwi subfamily proteins (PIWI). Animal piRNAs are 2′-O-methylated by HEN1 orthologs. In zebrafish, depletion of hen1 induces uridylation of piRNAs and facilitates decay, suggesting that methylation stabilizes piRNAs. However, the physiological significance of piRNA methylation in flies and mammals remains unclear. PIWI proteins are methylated at arginine residues (sDMA, symmetrical dimethyl arginine) at their N termini by orthologs of the methyl transferase PRMT5. In flies and mice, TDRD proteins interact with PIWI proteins through sDMA and may play important roles in piRNA metabolism.

Because U0126, an inhibitor of mitogen-activated protein kinase (MAPK), blocks the reduction of miR-124, the decay process may be dependent on the MAPK pathway. Of interest, a study on mammalian neuronal cells shows that most miRNAs turn over more rapidly in neurons than in other cell types (Krol et al., 2010). Neuronal activation accelerates decay of the miRNAs, whereas blocking neuronal activity stabilizes the miRNAs. It will be exciting to discover the nuclease(s) and the upstream signals for miRNA degradation in these systems.

Recently it has been shown that a polynucleotide phosphorylase (PNPase, a type I interferon-inducible 3′-to-5′ exonuclease) binds specifically to several miRNAs (miR-221, miR-222, and miR-106b) and induces rapid turnover in a human melanoma cell line (Das et al., 2010). Because there is no apparent commonality in terms of the sequences, it is unclear how PNPase recognizes the miRNAs specifically.

As mentioned above, there is substantial evidence linking uridylation/adenylation and exonucleolytic attack on small RNAs. A recent study provides evidence that extensive complementarity between a small RNA and its target RNA triggers uridyl/adenyl tailing as well as 3′→5′ trimming in flies and humans (Figure 1) (Ameres et al., 2010). Animal small RNAs with high complementarity to the targets, such as piRNAs and fly endo-siRNAs, appear to be generally protected by 2′-O-methylation at the 3′ end like plant small RNAs. It has been postulated that animal miRNAs, which do not carry methylation, maintain only partial complementarity with their targets so as to avoid tailing and trimming of miRNAs. Of note, viruses seem to exploit a related miRNA decay pathway to invade host cells more effectively. *Herpesvirus saimiri*, a family of primate-infecting herpesviruses, expresses viral noncoding RNAs called HSURs (*H. saimiri* U-rich RNAs). A recent report reveals that HSURs rapidly downregulate host miR-27 and that base-pairing between HSUR and miR-27 is required for the degradation (Cazalla et al., 2010). These discoveries imply an additional layer of stability control of small RNAs, which is influenced by the interaction with the target RNA.

miRNA Editing

Adenosine deaminases acting on RNAs (ADARs) convert adenosine to inosine on the dsRNA region of small RNA precursors (Figure 1 and Figure 3A). Because inosine (I) pairs with cytosine instead of uridine, such edits could alter the structure of small RNA precursor, thereby interfering with processing. For instance, editing of pri-miR-142 by ADAR1 and ADAR2 suppresses Drosha processing (Yang et al., 2006), whereas that of pre-miR-151 by ADAR1 interferes with Dicer processing (Kawahara et al., 2007a). Because hyperedited dsRNAs can be targeted by the nuclease Tudor-SN, RNA editing may also destabilize small RNA precursors (Scadden, 2005). In rare cases, RNA editing occurs in the seed sequence of miRNA, changing the targeting specificity. In the brain, where ADAR is abundant, miR-376 cluster miRNAs are frequently edited in the seed region and are redirected to repress a different set of mRNAs (Kawahara et al., 2007b). High-throughput sequencing of the fly endo-siRNA pool also reveals evidence for RNA editing (Kawamura et al., 2008). The precursors of endo-siRNAs (long hairpins and sense-antisense pairs) may be targeted by ADARs, although the functional significance of this siRNA modification is unknown.

Posttranslational Protein Modifications

Phosphorylation of RNase III Enzymes

Human Dicer interacts with two related dsRNA-binding proteins, TRBP and PACT. Although they do not influence Dicer processing itself, TRBP and PACT stabilize Dicer and may also function in RISC assembly (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). A recent study indicates that four serine residues of human TRBP (S142, S152, S283, and S286) are phosphorylated by the MAP kinase Erk, which controls cell proliferation, survival, and differentiation (Figure 1) (Paroo et al.,

2009). Phosphorylation enhances protein stability of TRBP, consequently elevating Dicer protein levels. Intriguingly, TRBP phosphorylation preferentially increases growth-promoting miRNAs such as miR-17, whereas tumor-suppressive let-7 is reduced. The mechanism of selective downregulation of let-7 is unclear, but it may be an indirect effect. An interesting implication of these findings is that the MAPK/Erk pathway exerts its effects, in part, by regulating miRNA biogenesis.

Drosha, a nuclear enzyme for pri-miRNA processing (Lee et al., 2003), has recently been shown to be a direct target of posttranslational modification (Tang et al., 2010). Mass spectrometry and mutagenesis studies reveal that human Drosha is phosphorylated at serine 300 (S300) and serine 302 (S302) (Figure 1). Phosphorylation of these residues is essential for the nuclear localization of Drosha and is required for pri-miRNA processing. Because both endogenous and overexpressed Drosha localize to the nucleus constitutively, it is unclear whether or not the phosphorylation at S300/S302 is a regulated process. Understanding the physiological significance of this regulation will require the identification of the kinase that phosphorylates Drosha.

Argonaute2 Is a Target of Multiple Modifications

Ago2 is subject to multiple posttranslational modifications (Figure 1). Human Ago2 binds to the type I collagen prolyl-4-hydroxylase (C-P4H(I)) that hydroxylates Ago2 at proline 700 (Qi et al., 2008). Depletion of C-P4H(I) reduces the stability of the Ago2 protein and, accordingly, downregulates siRNA-mediated silencing. Furthermore, hydroxylation is required for Ago2 localization to the processing body (P body), a cytoplasmic granule that is thought to be a site for RNA storage and degradation. P body localization of Ago2 is also enhanced by phosphorylation at serine 387, which is mediated by the p38 MAPK pathway (Zeng et al., 2008). However, given the controversy over the direct role of P body in small RNA-mediated silencing, the biological significance of P body localization of Ago2 remains unclear.

Ubiquitination also plays a part in the control of Ago2. Mouse Lin41 (mLin41 or Trim71), a stem cell-specific Trim-NHL protein, inhibits the miRNA pathway (Rybak et al., 2009). As an E3 ubiquitin ligase, mLin41 ubiquitinates Ago2 and targets it for proteasome-dependent degradation. Of interest, mLin41 is a target of let-7 miRNA, suggesting that mLin41 and let-7 may be engaged in a reciprocal negative feedback loop. Recently, other Trim-NHL proteins have been reported to associate with the Argonaute proteins and affect miRNA pathway. Mei-P26 (fly) inhibits miRNA biogenesis, whereas TRIM32 (mouse) and NHL-2 (worm) activate the miRNA pathway (Hammell et al., 2009; Neumüller et al., 2008; Schwamborn et al., 2009). Their mechanism of action appears to be different than that of mLin41 because the E3 ligase activity of Mei-P26 and TRIM32 is dispensable for their effects and because NHL-2 enhances miRNA activity without a change in miRNA levels.

Tudor Regulates PIWI Proteins

The PIWI (P element-induced wimpy testis) clade proteins bind to Piwi-interacting RNAs (piRNAs) and silence transposable elements in gonads. Mouse has three PIWI homologs (MILI, MIWI, and MIWI2), and there are three PIWI proteins in flies (Aubergine [Aub], AGO3, and Piwi) (Kim et al., 2009). Recent studies have revealed that PIWI proteins carry symmetrical dimethyl arginine (sDMA) at their N termini. Arginine methylation of PIWI is mediated by a methyl transferase PRMT5 (dPRMT5/capsuleen [csul]/dart5 in *Drosophila*) (Figure 3B) (Heo and Kim, 2009; Siomi et al., 2010). sDMA is recognized by Tudor domain-containing proteins (TDRDs), which are critical for germline development. In both flies and mice, deletion of TDRDs alters piRNA abundance and/or composition, indicating that TDRDs play important roles in the piRNA metabolism through specific binding to the sDMAs of PIWI proteins. How TDRDs act in the piRNA pathway at a molecular level awaits further investigation.

Perspectives

As we delve deeper and wider into the small RNA world, the emerging landscape becomes ever more complex on both the RNA and protein sides. High-throughput analyses have uncovered a considerable heterogeneity in small RNA populations. Some isomiRs are expressed differentially in certain tissues, suggesting that these variations may be associated with specific regulatory functions (Chiang et al., 2010). Biochemical and genetic studies also provide substantial evidence for the regulatory roles of the modifications discussed in this Review. Thus, it is likely that at least some of the observed heterogeneity reflects multiple layers of regulation. We should be cautious, however, in extrapolating the current evidence because it is unclear how much fraction of the small RNA and protein modifications translate into functional consequences and whether certain modifications simply reflect the noise of RNA metabolism.

In addition to the functionality issue, a number of key questions remain to be answered. Are there conserved pathways and enzymes for RNA and protein modifications? If so, what are the similarities and differences? 2′-O-methylation is applied to many small RNA pathways, but the details differ significantly in different systems. For instance, plant HEN1 acts on dsRNA duplexes, whereas animal HEN1 homologs methylate ssRNA loaded on Argonaute proteins. Uridylation/adenylation is carried out by a family of ribonucleotidyl transferases. How each member selectively recognizes its substrates is largely unknown. RNA stability is likely to play important roles in RNA silencing pathways. Decay pathways of small RNA are beginning to be unraveled, but there is no consensus between different species as yet. One possibility is that multiple enzymes act in parallel as in the mRNA decay pathway, which involves several 3′ exonucleases, 5′ exonucleases, and endonucleases. Some of the decay enzymes may function redundantly, and it remains one of the major challenges in the field to identify them. Protein modification is also emerging as one of the key regulatory layers. Outstanding questions include which enzymes are involved, what the in vivo significance of such modifications is, and whether the protein modifications are developmentally regulated. Future studies will reveal new types of modifications, additional regulatory factors, and their biological relevance.

The RNA silencing machinery should respond accurately to developmental and environmental cues. Most signaling pathways are thought to be connected to RNA silencing, but we are just beginning to understand the molecular links between RNA silencing and cell signaling. What the upstream signals are, how certain RNAs and proteins get specifically recognized,

and what the downstream effects of the modifications are await elucidation. We also need to understand the interplay between different modifications. There appears to be a crosstalk between certain modifications of RNA (such as methylation, uridylation, and decay), which may influence their fate and function. It is likely that there is a crosstalk between the different posttranslational modifications in the proteins involved in the biogenesis and effector functions of small RNA silencing pathways. Understanding these networks will undoubtedly provide ample opportunities to manipulate RNA silencing and will reveal new lessons about gene regulation.

ACKNOWLEDGMENTS

We thank members of V.N.K.'s laboratory for helpful discussions and comments. This work was supported by the Creative Research Initiatives Program (20100000021) and the National Honor Scientist Program (20100020415) through the National Research Foundation of Korea (NRF) and the BK21 Research Fellowships (I.H.) from the Ministry of Education, Science and Technology of Korea. We apologize to authors whose work has not been covered because of space limitations.

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